PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/70 — A1

(11) International Publication Number: **WO 97/46716**

(43) International Publication Date: 11 December 1997 (11.12.97)

(21) International Application Number: PCT/IT97/00128

(22) International Filing Date: 3 June 1997 (03.06.97)

(30) Priority Data:
RM96A000404 7 June 1996 (07.06.96) IT

(71) Applicant *(for all designated States except US)*: WABCO B.V. [NL/NL]; Industrieweg 4, P.O. Box 1074, NL-7940 KB Meppel (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BOSIO, Paolo [IT/IT]; Sorin Biomedica Diagnostics s.p.a., Via Crescentino, I-13040 Saluggia (IT). STRUMIA, Claudia [IT/IT]; Sorin Biomedica Diagnostics s.p.a., Via Crescentino, I-13040 Saluggia (IT). CLEMENZA, Filippo [IT/IT]; Sorin Biomedica Diagnostics s.p.a., Via Crescentino, I-13040 Saluggia (IT).

(74) Agents: BANCHETTI, Marina et al.; Ing. Barzanò & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD TO DETECT HCV SPECIFIC NUCLEIC ACIDS

(57) Abstract

A process to detect hepatitis C virus (HCV) specific nucleic acids comprising the steps of: (a) reverse transcribing the viral RNA by means of a primer having specified sequences; (b) amplifying by a single polymerase chain reaction (PCR) with a specific primer; wherein the Mg^{++}/Taq polymerase ratio in the reaction mix is of approx. 100 nmoles/enzyme unit; (c) revealing the amplified product by means of the DEIA method using a specific probe.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

METHOD TO DETECT HCV SPECIFIC NUCLEIC ACIDS

The invention concerns a method to detect hepatitis C virus specific (HCV) nucleic acids.

In particular the invention refers to an improved method to detect HCV amplified DNA, by means of a single step polymerase chain reaction (PCR), under controlled and optimized reaction parameters, and of a revealing system of amplified products.

One of the most used methods to detect HCV specific nucleic acids is based upon the reverse transcription of viral RNA to cDNA, followed by a double amplification step (nested PCR) of the most conserved genome region (5'UTR). The amplified product of the second amplification step may be identified by means of revealing techniques as electrophoresis or enzyme mediated signals. The double amplification allows to reach a very high sensitivity able to identify even few viral RNA molecules. On the other hand the double PCR step has many disadvantages mainly due to DNA contamination from previous amplifications, length of time, high costs.

In order to overcome said disadvantages there is the need to set up a single step amplification protocol, which reaches similar levels of sensitivity than the nested PCR.

The authors of the instant invention have optimized the nested PCR reaction conditions in order to eliminate the second step. Moreover the system used to reveal amplified products is the DNA Enzyme Immunoassay (DEIA), which mekes the use of a specific capturing probe and of a monoclonal antibody able to recognize double strand DNA (Mantero G. et al. Clin Chem. 37, 422-429, here incorporated by references).

The combination of all of optimized parameters, both of the amplification and of the revealing step, allowed to set up a method able to detect HCV specific DNA with a sensitivity equal, if not higher than the nested PCR, but with no disadvantages.

In order to optimize the PCR reaction parameters a comparison of first (not revealed by DEIA assay) and second (revealed by DEIA assay) amplification step, and an analysis to individuate the capturing probe with best features for a revealing step by DEIA assay as well, were performed.

C. Payan et al. J. Virol. Meth. (1995) 53, 167-175 describe a process to reveal HCV nucleic acids characterised by a single step for both the reverse transcription and the amplification. The amplification is a single step, no nested-PCR. Primer sequences are deduced from the HCV genome nucleotide sequence, and in particular from the high conserved 5'end region. The magnesium chloride final concentration in the PCR solution is of 1 mM, the ratio $[Mg^{++}]$/U Taq being 35 nmol/U Taq.

C. Payan et al. Res. Virol. (1995) 146, 363-70 optimize the above referred process by modifying the $MgCl_2$ concentration to 2 mM, the units of Mu-MLV RNAse and of Taq polymerase, to 10 U and 1 U respectively, with a ratio of 42 nmol $[Mg^{++}]$/U Taq, and thus diminishing the RNA copy number to be revealed from 15 to 10.

It is therefore evident that even minimal variations of general reaction conditions may interfere and increase significantly the yield and sensitivity.

It is an object of the instant invention a method to detect hepatitis C virus (HCV) specific nucleic acids comprising the steps of:

a) reverse transcribing the viral RNA with a primer having a sequence substantially homologous to one of the sequences SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3;

b) amplifying by means of a single step polymerase chain reaction (PCR) wherein the primer has a sequence substantially homologous to sequences of SEQ ID No. 4 or SEQ ID No. 5, wherein the ratio between the Mg^{++} ion concentration and of Taq polymerase in the reaction mix is of approximately of 100 nmoles/enzyme unit;

c) revealing the amplified product by means of DEIA method using a probe having the sequence of SEQ ID No. 6, or a probe having a sequence substantially homologous or complementary thereof.

It is a further object of the invention the use of the of an oligonucleotide of SEQ ID No. 6 or of an oligonucleotide having a sequence substantially homologous or complementary thereof as probe for revealing HCV specific nucleic acids by means of the DEIA method.

It is a further object of the invention a composition to reveal HCV specific nucleic acids by means of the DEIA method comprising an oligonucleotide of SEQ ID No. 6 or of sequence substantially homologous or complementary thereof.

By comparing the two steps of the nested PCR, as in Table 2, it is clear that main differences are: 1) primer sequences; 2) $MgCl_2$ concentration (which is a Taq polymerase activator); 3) buffer concentration (TRIS-HCl pH 8.3); 4) solution ionic strength (mainly related to the KCl concentration); 5) deoxinucleotidetriphosphate concentration (dNTP). Primer sequences are shown in Table 1a.

Table 1a

Primer sequences

name	sequence	SEQ ID No.
1 CH	5' GGT GCA CGG TCT ACG AGA CCT 3'	SEQ ID No. 1
2 CH	5' AAC TAC TGT CTT CAC GCA GAA 3'	SEQ ID No. 4
1 TS	5' GCG ACC CAA CAC TAC TCG GCT 3'	SEQ ID No. 2
4 CH	5' ATG GCG TTA GTA TGA GTG 3'	SEQ ID No. 5
PT2	5' CGG TGT ACT CAC CGG TTC 3'	SEQ ID No. 3
PKY78	5' CTC GCA AGC ACC CTA TCA GGC AGT 3'	SEQ ID No. 7
PKY80	5' GCA GAA AGC GTC TAG CCA TGG CGT 3'	SEQ ID No. 8

Probe sequences for the revealing step are shown in Table 1b.

Table 1b

3 CH	5' CGG TGA GTA CAC CGG AAT TGC CAG GAC CGG GTC CTT TCT 3'	SEQ ID No. 9
WT	5' GCC ATA GTG GTC TGC GG 3'	SEQ ID No. 10
PT 21	5' GGG AGA GCC ATA GTG GTC TGC 3'	SEQ ID No. 6
KY 150	5' CAT AGT GGT CTG CGG AAC CGG TGA GT 3'	SEQ ID No. 11
HCV40	5' CCA TAG TGG TCT GCG GAA CCG TGA GTA CA 3'	SEQ ID No. 12
CH5	5' TAG TGG TCT GCG GAA CCG GT 3'	SEQ ID No. 13

Table 2

Two step nested PCR parameters

param.	I step	II step
primer	1CH(50pmol)/2CH(50pmol)	1TS(50pmol)/4CH(50pmol)
[Tris-HCl]	22.5 mM	10 mM
[KCl]	62.5 mM	50 mM
[$MgCl_2$]	4 mM	1.5 mM
[dNTP]	260 µM **	200 µM
Taq pol.	2.5 U	2.5 U

** The concentration accounts also for added dNTPs during the reverse transcription step.

1) SAMPLE PREPARATION

Sera to be tested by nested or single step PCR were treated to isolate RNA. Commercially available products, as RNAzol B e ULTRASPEC (Biotecx), were used following instructions of producer.

2) NESTED PROTOCOL

The nested PCR to detect HCV RNA at the 5'UTR region was used as control. 5 µl of extracted RNA were reverse transcribed in 25 µl volume containing 22.5 mM TRIS-HCl pH 8.3, 62.5 mM KCl, 4 mM $MgCl_2$, 250 µM dNTPs, 4U AMV-RT, 2 µM 1CH antisense primer, 25 U RNAse inhibitors (HRPI). The reverse transcription was performed at 42°C for 1 hr and the enzyme was further denatured at 100°C for 10 min. The nested PCR first step was performed in a 100 µl volume containing cDNA (25 µl from the reverse transcription), 22.5 mM TRIS-HCl pH 8.3, 62.5 mM KCl, 4 mM $MgCl_2$, 200 µM dNTPs (only for this step), 0.5 µM 2CH sense primer, 2.5 U Taq polymerase; thermal cycle: 94°C 1 min., 50°C 1 min., 72°C 2 min., 35 cycles. For the second step 3 µl from the first step were amplified in a 100 µl volume containing 10 mM TRIS-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM 1TS internal antisense primer, 0.5 µM 4CH internal sense primer; 2.5 U Taq polymerase; thermal cycle: 94°C 1 min., 50°C 1 min., 72°C 2 min.; 25 cycles.

20 µl of each amplified product were tested by the DEIA assay using as probe the 3CH oligonucleotide.

Experimental tests are hereinbelow reported, according to different parameters of the first and second PCR steps. In all of reactions the amount of AMV-RT enzyme was of 15 U/sample.

3) CHANGE of PRIMER SEQUENCES

Different combinations of primers were assayed in the single step RT-PCR.

Nested external primers: 1CH (antisense) / 2CH (sense)
Nested internal primers: 1TS (antisense) / 4CH (sense)
Nested external/internal primers: 1CH (antisense) / 4CH (sense)
Nested internal/external primers: 1TS (antisense) / 2CH (sense)

The antisense primer PT2 and those described in the EP 529493 application, identified as PKY78 antisense and PKY80 sense, were also assayed.

Reaction conditions were those for all of primer combinations and are as follows:

- reverse transcription was performed as for the nested protocol, but of AMV-RT units;
- PCR was performed in 100 µl final volume containing cDNA, 22.5 mM TRIS-HCl pH 8.3, 62.5 mM KCl, 4 mM $MgCl_2$, 200 µM dNTP (only those added in this step), 0.5 µM sense primer (according to different combinations), 2.5 U Taq polymerase; thermal cycle: 94°C 1 min., 50°C 1 min., 72°C 2 min.; 45 cycles;
- 20 µl of each amplified product were assayed by means of the DEIA Enzyme Immunoassay with the 3CH probe.

By only varying primer combinations 1CH, 2CH, 4CH, 1TS, PT2 no detectable amplified product was made in a single step PCR amplification. Even when "alternative" sequences were use (primers as in EP 529493) no clear positive results were obtained (positive result only in one sample), see Table 3 (positive values in bold character).

Table 3

sample	nested PCR	single step PCR primer combination				
		1CH/2CH	1TS/4CH	1CH/4CH	1TS/2CH	PKY78/PKY80
	O.D. 450/630 nm					
0 RNA	0,061	0,068	0,076	0,176	0,110	0,084
N. 1 Neg	0,084	0,062	0,067	0,103	0,112	0,089
N. 2 Pos	**2,185**	0,123	0,078	0,108	0,103	**1,159**
N. 3 Pos	**1,979**	0,066	0,081	0,109	0,109	0,092
N. 4 Pos	**2,566**	0,054	0,084	0,105	0,113	0,069
cut-off	0,192	0,192	0,192	0,231	0,231	0,220

4) MODIFICATIONS OF $MgCl_2$ CONCENTRATION

It has been found that the $MgCl_2$ concentration (a Taq polymerase activator) has to be finely modulated to obtain the best yield of the amplification reaction. During the amplification, $MgCl_2$ concentrations of 1.5 mM, 2.5 mM, 4 mM, corresponding, respectively, to 60nmol, 100nmol, 160nmol of Mg^{++} per Taq unit were used. The reverse transcription reaction was performed as in the nested PCR protocol, but of enzyme units. The PCR reaction was performed in a 100 µl volume containing cDNA (25 µl from the reverse transcription mix), 22.5 mM TRIS-HCl pH 8.3, 62.5 mM KCl, $MgCl_2$ at different concentrations, 200 µM dNTPs (only dNTPs added in this step), 0,5 µM 2CH primer and 2,5 U Taq polymerase.

Data obtained by means of the DEIA immunoenzyme assay (with the 3CH probe), as in Table 4, show that:

a) very low $MgCl_2$ concentrations (1.5 mM, 60 nmol/U) reduce the yield of the single step PCR reaction; in fact, only 50% of nested positive samples are still positive;

b) very high $MgCl_2$ concentrations (4 mM, 160 nmol/U) are not workable since no nested positive sample was confirmed as such in the single step reaction;

c) a $MgCl_2$ concentration of 2.5 mM (100 nmol/U) is the best, confirming all of data obtained by the nested PCR.

Table 4

sample	nested PCR	single step PCR [$MgCl_2$] 1,5 mM	2,5 mM	4,0 mM
	O.D. 450/630 nm			
N 1	**2,085**	**2,013**	**2,017**	0,065
N 2	**1,622**	0,145	**0,913**	0,092
N 3	**2,107**	**1,797**	**1,912**	0,071
N 4	**2,163**	0,115	**0,261**	0,080
N 5	**2,194**	**0,274**	**1,092**	0,078
N 6	**0,899**	0,117	**0,560**	0,071
N 7	0,107	0,122	0,158	0,066
N 8	0,055	0,115	0,131	0,077
N 9	0,054	0,115	0,140	0,077
N10	0,051	0,102	0,157	0,063
N11	0,084	0,155	0,134	0,062
cut off	0,253			

5) MODIFICATIONS OF REACTION BUFFER CONCENTRATION

As opposite to $MgCl_2$, differences in the concentration of TRIS-HCl (12.5 mM instead of 22.5 mM) and KCl (50 mM instead of 62.5 mM) do not interfere in a significant way with the efficacy of the single step amplification, as DEIA obtained data are comparable to controls, as in Table 5.

Table 5

SAMPLE	Tris-HCl 12,5 mM KCl 12,5 mM	Tris-HCl 22,5 mM KCl 62,5 mM
	O.D. 450/630 nm	
N°2368	0,900	0,912
N°2369	1,012	1,038
N°2370	1,064	1,311
N°2381	0,685	1,604
N°2397	0,549	0,508
N°2404	0,028	0,054
N°2411	1,354	1,145
N°2412	1,465	1,780
N°2416	1,387	1,797
N°2452	0,845	1,514
N°2464	0,872	1,175
N°2487	1,032	2,274
cut off	0,200	0,200

Reverse transcription and amplification reactions were performed as described in 4).

6) MODIFICATION OF dNTP CONCENTRATION

Experimental tests were performed in order to evaluate the nucleotide concentration during the single step amplification. Results obtained by DEIA assays (as in Table 6) show that a dNTP concentration lower than the single step optimized concentration (83 µM instead of 200 µM, as referred to dNTP added to the amplification step) lowers the reaction yield.

Table 6

SAMPLE	83 µM dNTP	200 µM dNTP
	O.D. 450/630 nm	
N° 2364	0,351	1,140
N° 2368	0,240	0,900
N° 2369	0,610	1,012
N° 2370	0,768	1,064
N° 2383	0,131	0,580
N° 2397	0,160	0,549
N° 2400	0,114	0,942
N° 2411	0,105	1,354
N° 2412	0,126	1,465
N° 2413	0,309	1,150
N° 2416	0,239	1,387
cut off	0,270	0,270

Reverse transcription and amplification reactions were performed as described in 4).

7) SINGLE STEP PCR

According to the above described tests the best reaction conditions to amplify HCV RNA are as follows:

A) REVERSE TRANSCRIPTION

5 µl of RNA were reverse transcribed in a 25 µl volume containing 50 mM TRIS-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 250 µl dNTP, 2 µM 1CH antisense primer, 25 U HRPI and 15 U AMV-RT. The reverse transcription reaction was performed at 42°C for 1 hr, followed by an enzyme denaturation step at 100°C for 10 min.

B) AMPLIFICATION

The amplification reaction was performed in a 100 µl volume containing the cDNA from the reverse transcription step (25 µl), 22.5 mM TRIS-HCl pH 8.3, 62.5 mM KCl, 2.5 mM $MgCl_2$, 0.5 µM 2CH sense primer, 200 µM

dNTP (referring only to dNTP added during said step, and 2.5 U Taq polymerase.

C) THERMAL CYCLE

94°C 1 min - 50°C 1 min - 72°C 2 min 45 cycles

8) REVEALING PROBE

In all of above experiments the 3CH revealing probe was used (Sorin Biomedica Diagnostics SpA PS000C Hepatitis C). Different probes were tested, having sequences deduced from a different region of the 5' UTR terminus. Various parameters were evaluated, as the oligonucleotide length that may allow an efficient hybridisation to the complementary sample, and a sequence excluding the formation of thermodynamically stable loops or dimers, due to the presence of internal homologous stretches, as well. In order to select the optimal sequence an analysis of thermodynamic features of some oligonucleotides, as derived from the internal region of the amplified sequence (HCV genome 5' UTR region) was performed.

Table 7

probe	ΔG hybrid	ΣΔG loops	ΣΔG dimers	ΣΔG (loops + dimers)
PROBE	Kcal/mol			
WT	- 29,6	1,3	0,0	1,3
PT 21	- 35,5	1,1	0,0	1,1
KY150	- 45,7	- 0,6	- 7,4	- 8,0
HCV40	- 52,9	- 0,3	- 7,4	- 7,7
CH 5	- 35,9	- 0,3	- 7,4	- 7,7
3 CH	- 83,1	- 8,1	no dimers	- 8,1

Some of the selected sequences were deduced from prior art literature, others designed to satisfy the above requisites. The thermodynamic analysis was performed with the OLIGO.EXE structure © (ver 3.3) program distributed by MedProbe A.S. (Norvegy), by

maintaining as constant two parameters: the probe (30 nM) and the salt (188 mM) concentration. These parameters are those experimentally used during the DEIA assay.

The thermodynamic analysis of sequences, as in Table 7, shows that hybridisation reactions of all of oligos with complementary sequences are thermodynamically favoured. As expected the lowest ΔG values are those of the longest probes (3CH, HCV40, KY150), which on the other hand favour the formation of either loops or dimers. On the other side, among the shortest probes, PT21 and WT are those that, according to their sequence typology, show the best features, whereas CH5, though having analogous dimensions to the other two probes, may give rise to undesired thermodynamically stable dimers. The PT21 probe is able to form a more stable specific hybrid than the WT probe. The two PT21 and 3CH probes were then compared. The reaction conditions were standard conditions of the DEIA kit (Sorin Biomedica Diagnostics SpA, GEN-ETI-K-DEIA cod. PS0001) to reveal amplified HCV and foresee a specific hybridisation at 50°C. Experimental results, reported in Table 8, confirm that the PT21 probe allows a more efficient hybridisation with the amplified complementary strand. Approximately 96% of analyzed positive samples show absorbance values higher than 1,0 O.D., whereas with the 3CH probe only 40% of samples overcomes this threshold.

Table 8

sample	probe	
	3CH	PT21
	O.D. 450/630 nm	
N 1	0,633	2,259
N 2	0,215	1,430
N 3	0,579	2,105
N 4	0,610	2,361
N 5	0,230	0,560
N 6	0,833	2,051
N 7	1,734	2,926
N 8	1,872	2,852
N 9	0,574	2,546
N 10	1,147	2,829
N 11	1,562	2,788
N 12	1,206	2,897
N 13	2,002	2,876
N 14	0,499	2,253
N 16	1,600	2,715
N 17	1,522	2,656
N 18	2,281	2,682
N 19	0,690	2,238
N 20	0,275	2,274
cut off	0,190	0,198

Finally, experimental analysis of PT21 and 3CH probes were performed by evaluating their specificity in the DEIA conditions as above. The analytical specificity was determined with probe unrelated DNA samples, which are representative of molecular dimensions and of sequence heterogeneity: different concentrations of salmon sperm DNA; different concentrations of calf thymus DNA; unrelated amplified DNA. Results, as reported in Table 9, did not show significant aspecific reactions,

being absorbance values lower than cut-off (cut-off = average absorbance of negative samples + 0.150 O.D., as suggested in the DEIA assay).

Table 9

SPECIFICITY PT21 - 3CH

SAMPLE	PT21 SPECIFICITY	3CH SPECIFICITY
	GEN.ETI.K HCV REAGENT LOT GEN.ETI.K DEIA #7400110 O.D. 450/630 nm	
PCR HIV	0,043	0,050
	0,051	0,059
PCR HDV	0,057	0,105
	0,078	0,112
TV 1 µg/µl	0,079	0,058
	0,058	0,058
TV 0,8 µg/µl	0,069	0,085
	0,068	0,066
TV 0,4 µg/µl	0,061	0,053
	0,062	0,056
TV 0,2 µg/µl	0,058	0,051
	0,080	0,051
TV 0,1 µg/µl	0,061	0,053
	0,059	0,048
TV 0,05 µg/µl	0,065	0,048
	0,055	0,051
SS 1 µg/µl	0,071	0,059
	0,069	0,058
SS 0,8 µg/µl	0,065	0,055
	0,062	0,053
SS 0,4 µg/µl	0,054	0,053
	0,055	0,052
SS 0,2 µg/µl	0,058	0,046
	0,053	0,049
SS 0,1 µg/µl	0,061	0,047
	0,052	0,048

SS 0,05 µg/µl	0,061 0,045	0,049 0,059
average neg. ctrl.	0,049	0,047
average pos. ctrl.	2,103	2,331
cut off	0,199	0,197

TV: new-born calf time DNA; SS: salmon sperm DNA.

The setting of the single step DNA HCV amplification and revealing protocol was performed by optimizing of amplification reaction conditions (in particular by changing the $MgCl_2$ concentration), and by using a new revealing probe (PT21). Said approach was able to give results comparable to the NESTED plus DEIA method.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: SORIN BIOMEDICA DIAGNOSTICS S.p.A.
(B) STREET: Via Borgonuovo 14
(C) CITY: Milan
(E) COUNTRY: Italy
(F) POSTAL CODE (ZIP): 20121

(ii) TITLE OF INVENTION: Method to detect HCV specific nucleic acids

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGTGCACGGT CTACGAGACC T 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCGACCCAAC ACTACTCGGC T 21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGGTGTACTC ACCGGTTC 18

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AACTACTGTC TTCACGCAGA A 21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATGGCGTTAG TATGAGTG 18

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGGAGAGCCA TAGTGGTCTG C 21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTCGCAAGCA CCCTATCAGG CAGT 24

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCAGAAAGCG TCTAGCCATG GCGT 24

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CGGTGAGTAC ACCGGAATTG CCAGGACGAC CGGGTCCTTT CT 42

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCCATAGTGG TCTGCGG 17

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CATAGTGGTC TGCGGAACCG GTGAGT 26

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCATAGTGGT CTGCGGAACC GTGAGTACA 29

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TAGTGGTCTG CGGAACCGGT 20

CLAIMS

1. Process to detect hepatitis C virus (HCV) specific nucleic acids comprising the steps of:

a) reverse transcribing the viral RNA by means of a primer having a nucleotide sequence substantially homologous to SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3;

b) amplifying by a single polymerase chain reaction (PCR) wherein the primer has a nucleotide sequence substantially homologous to SEQ ID No. 4 SEQ ID No. 5; wherein the Mg^{**} / Taq polymerase ratio in the reaction mix is of approx. 100 nmoles/enzyme unit;

c) revealing the amplified product by means of the DEIA method using as probe an oligonucleotide of SEQ ID No. 6, or having a sequence substantially homologous or complementary thereof.

2. Use of the oligonucleotide of SEQ ID No. 6 or having a sequence substantially homologous or complementary thereof as probe to reveal HCV specific nucleotides by means of the DEIA method.

3. Composition to reveal HCV specific nucleotides with the DEIA assay comprising the oligonucleotide of SEQ ID No. 6 or an oligonucleotide of sequence substantially homologous or complementary thereof.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IT 97/00128

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 19743 A (CHIRON CORP) 12 November 1992 page 140, seq id 126; page 138, seq id 123 ---	1
X	WO 95 06753 A (US GOVERNMENT) 9 March 1995 see the whole document ---	1
X	EP 0 529 493 A (HOFFMANN LA ROCHE) 3 March 1993 see the whole document ---	1-3
X	IMBERTI L. ET AL.,: "An immunoassay for specific amplified HCV sequences" J. VIROLOGICAL METHODS, vol. 34, - 1991 pages 233-243, XP002042919 see the whole document --- -/--	2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 October 1997	22.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

CLAIMS

1. Process to detect hepatitis C virus (HCV) specific nucleic acids comprising the steps of:

a) reverse transcribing the viral RNA by means of a primer having a nucleotide sequence substantially homologous to SEQ ID No. 1, SEQ ID No. 2 or SEQ ID No. 3;

b) amplifying by a single polymerase chain reaction (PCR) wherein the primer has a nucleotide sequence substantially homologous to SEQ ID No. 4 SEQ ID No. 5; wherein the Mg^{++} / Taq polymerase ratio in the reaction mix is of approx. 100 nmoles/enzyme unit;

c) revealing the amplified product by means of the DEIA method using as probe an oligonucleotide of SEQ ID No. 6, or having a sequence substantially homologous or complementary thereof.

2. Use of the oligonucleotide of SEQ ID No. 6 or having a sequence substantially homologous or complementary thereof as probe to reveal HCV specific nucleotides by means of the DEIA method.

3. Composition to reveal HCV specific nucleotides with the DEIA assay comprising the oligonucleotide of SEQ ID No. 6 or an oligonucleotide of sequence substantially homologous or complementary thereof.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IT 97/00128

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 518 313 A (MITSUBISHI CHEM IND) 16 December 1992 see the whole document	1
Y	MANTERO G ET AL: "DNA ENZYME IMMUNOASSAY: GENERAL METHOD FOR DETECTING PRODUCTS OF POLYMERASE CHAIN REACTION" CLINICAL CHEMISTRY, vol. 37, no. 3, pages 422-429, XP000371646 cited in the application see the whole document	1-3
Y	PATENT ABSTRACTS OF JAPAN vol. 096, no. 002, 29 February 1996 & JP 07 250700 A (TONEN CORP;OTHERS: 02), 3 October 1995, see abstract	1-3
Y	EP 0 699 751 A (MITSUBISHI CHEM CORP) 6 March 1996 see seq id 38, page 42 see the whole document	1-3
Y	IMBERTI L. ET AL.,: "Non-radioisotopic methods for DNA probes" ANN. BIOL. CLIN., vol. 50, - 1992 pages 163-167, XP002042920 see the whole document	1-3
A	GUNJI T ET AL: "SPECIFIC DETECTION OF POSITIVE AND NEGATIVE STRANDED HEPATITIS C VIRAL RNA USING CHEMICAL RNA MODIFICATION" ARCHIVES OF VIROLOGY, vol. 134, no. 3/04, pages 293-302, XP000615872 see the whole document	1-3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IT 97/00128

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9219743 A	12-11-92	AU 668355 B	02-05-96
		AU 2155892 A	21-12-92
		BG 98200 A	31-01-95
		CZ 9601210 A	14-08-96
		CZ 9302377 A	13-04-94
		EP 0585398 A	09-03-94
		HU 69609 A	28-09-95
		JP 6508026 T	14-09-94
		NO 934019 A	05-11-93
		PL 169880 B	30-09-96
		PL 170151 B	31-10-96
		SK 123293 A	08-06-94
WO 9506753 A	09-03-95	AU 7718594 A	22-03-95
EP 0529493 A	03-03-93	AU 665952 B	25-01-96
		AU 2121992 A	04-03-93
		CA 2076793 A	28-02-93
		CN 1071512 A	28-04-93
		EP 0787807 A	06-08-97
		JP 6086700 A	29-03-94
		NZ 244071 A	27-01-95
		US 5527669 A	18-06-96
		US 5580718 A	03-12-96
		ZA 9206283 A	01-03-93
EP 0518313 A	16-12-92	CA 2070952 A	12-12-92
		JP 6000085 A	11-01-94
		JP 6000086 A	11-01-94
EP 0699751 A	06-03-96	JP 6311885 A	08-11-94
		CA 2104649 A	26-02-94